epinephrine from adrenergic fibers and by blocking its reuptake; at higher concentrations, AMI acts as an antagonist by blocking alpha-adrenergic receptors. The results do not establish the mechanism by which AMI alters *PS* in primates, but in rodents we showed that they are mediated by the central adrenergic system (*1*). The AMI-induced increase in the brain uptake of water is abolished either by pretreatment with 6-hydroxydopamine (intraventricularly) or phenoxybenzamine. The cerebrovascular effects of AMI are not mediated by the central serotonin, antimuscarine, or antihistamine actions of the drug.

AMI also induced a decrease in cerebral metabolic rate (Fig. 2), an observation supported by the report (*8*) that desipramine alters cerebral glucose utilization in rats at a dose and time which we have shown increase the brain uptake of water in rodents (*1*). Other investigators (*9*) reported that stimulation of the locus coeruleus and iontophoretic application of norepinephrine altered the responsivity of neurons in somatosensory cortex and lateral geniculate body to both excitatory and inhibitory afferent input, suggesting that the central adrenergic system may modulate transmission of information to these and possibly other neural systems.

All these findings are consistent with a system which acts to tonically influence cerebral function by regulating cerebral homeostasis. A system capable of adjusting the sensitivity of neurons to excitatory or inhibitory input could probably also affect cerebral oxygen utilization and the cerebromicrocirculation. Regulation of cerebromicrovascular permeability and cerebral metabolic rate is essential for normal cerebral functioning. The cerebral microenvironment must remain constant despite systemic changes in hydrostatic pressure and plasma osmolarity as well as changes in extracellular fluid within the brain due to regional metabolic shifts. Neural regulation would provide an additional means by which the brain can compensate for such changes. Substances whose passage across the blood-brain barrier may be similarly regulated could include electrolytes and metabolic substrates.

Whether these findings are relevant to the clinical use of antidepressants is not yet clear. However, the AMI-induced increase in *PS* occurs in primates as well as rodents and is observed in both species at concentrations which are therapeutic for human patients (*6*). Moreover, the central adrenergic system, which appears to mediate the *PS* effect of antidepressant drugs, is the neurotransmitter system thought to be involved in the pathophysiology of major mood disorders.

SHELDON H. PRESKORN
Department of Psychiatry and Pharmacology, University of Kansas School of Medicine, Kansas City 66103

MARCUS E. RAICHLE
BOYD K. HARTMAN
Departments of Neurology, Neurosurgery, and *Psychiatry* and *Mallinckrodt Institute of Radiology, Washington University School of Medicine, St. Louis, Missouri 63110*

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Measurement of Intracellular Free Calcium in Monkey Kidney Cells with Aequorin

Abstract. *A method has been developed for the measurement of intracellular free calcium in mammalian cells. The calcium-sensitive photoprotein aequorin can be incorporated into isolated cells by hypo-osmotic treatment without altering the cell viability, permeability, or metabolism. Intracellular calcium activity (Ca_i^{2+}) was monitored in a perfusion system. In monkey kidney cells (LLC-MK$_2$), Ca_i^{2+} is approximately 57 nanomoles per liter. Changes in Ca_i^{2+} with time can also be followed: exposure of the cells to anaerobiosis or the calcium ionophore A23187 reversibly increases Ca_i^{2+}. The method has also been successfully tested in rat hepatocytes.*

Until now, it has been exceedingly difficult to measure cytosolic free calcium (Ca_i^{2+}) in mammalian cells. This difficulty has been a great obstacle in our understanding of the control, modulation, and regulation of cell calcium and of many calcium-dependent cellular functions (*1*). The calcium-sensitive photoprotein aequorin has been successfully injected into giant cells such as the squid axon and the barnacle muscle to measure Ca_i^{2+} (*2*). But the incorporation of aequorin or obelin, another calcium-sensitive photoprotein, into small mammalian cells has proved difficult or has been limited to virus-infected cell hybrids (*3*).

Table 1. Intracellular concentration of free calcium in cultured monkey kidney cells.

Experiment number	L (nA)	L_{max} (mA)	pF	Ca_i^{2+} (nM)
1	3.60	1.09	5.48	56
2	1.70	1.74	6.01	16
3	0.80	0.20	5.40	62
4	0.84	0.64	5.88	28
5	2.0	1.64	5.91	25
6	8.5	2.2	5.40	62
7	7.5	1.18	5.20	76
8	0.52	0.08	5.19	80
9	5.50	0.101	5.26	75
10	0.53	0.05	4.98	90
Mean ± standard error				57.0 ± 8.0

Aequorin is well suited to measure Ca_i^{2+} since its interaction with calcium is fast enough (time constant, 5 milliseconds) to reflect changes in Ca_i^{2+}. It is sensitive to Ca_i^{2+} and much less sensitive to intracellular free magnesium (Mg_i^{2+}), and it does not appear to bind to intracellular structures or alter intracellular functions (*4*). We have developed a method which incorporates enough aequorin to permit the detection of a calcium-dependent signal in suspensions of small mammalian cells and yet leaves the cells demonstrably intact and viable. We call this method the hypo-osmotic shock treatment (HOST).

Cultured monkey kidney cells (LLC-MK$_2$), grown as monolayers in Eagle's

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minimum essential medium, were harvested from roller bottles with a soft rubber policeman. The cells were washed twice with a buffer consisting of 120 m*M* NaCl and 3 m*M* *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (Hepes) (*p*H 7.4) at 4°C. The washing of the cells in cold, calcium-free medium is known to rapidly reduce the calcium bound to the cells (*5*). The reduction of cellular calcium is necessary to avoid the ignition (activation) of aequorin during incorporation. A volume of 0.5 ml of washed cells, packed by centrifugation at 800*g*, was then transferred into a polyethylene test tube containing 2 ml of a buffered hypo-osmotic solution of 3 m*M* Hepes (*p*H 7.4), 3 m*M* magnesium adenosine 5′-triphosphate, and 1 to 10 μg of aequorin and allowed to remain there for 2 minutes at 4°C. During exposure to this hypo-osmotic solution, the surface membrane apparently becomes permeable to aequorin which then enters the cells. After 2 minutes, the normal osmolarity was reestablished by the addition of 0.145 ml of a 2*M* KCl, 3 m*M* Hepes solution. With a normal osmolarity, the surface membrane again becomes impermeable to aequorin, thus trapping some of the photoprotein inside the cells. The cells were kept in the cold KCl solution for 15 minutes and then washed several times with a Krebs-Henseleit bicarbonate (KHB) buffer containing 1.3 m*M* calcium at 37°C and saturated with a mixture of 95 percent O_2 and 5 percent CO_2. During this step, the extracellular aequorin is ignited and washed away.

After 1 hour in KHB, the cells were transferred to a 5-ml (10 by 40 mm) glass cuvette. The cells were placed within a glass wool plug loosely packed in the lower half of the cuvette. The cells readily attached to the glass wool and they were perfused from below at a rate of 0.6 ml/min without loss of cells from the cuvette into the effluent, as measured in terms of the protein content of the perfusate by the Lowry method (*6*). The cuvette was introduced into a light-recording apparatus (Fig. 1), designed to maximize the collection of light from the sample to the photocathode of a photomultiplier tube (EMI model 9635A). It is composed of two ellipsoidal reflectors (Melles Griot, Irvine, California). The photocathode of the photomultiplier tube is located at one focus and the cuvette at the other. The cuvette containing the cell suspension is positioned vertically over the photomultiplier. The current signal from the photomultiplier tube is amplified with an analog device (*4*) and displayed on a Recordall strip chart (Fisher).

Changes in Ca_i^{2+} can be measured in cells containing aequorin as shown in Figs. 2 and 3. In the experiment illustrated in Fig. 2, the cells were suddenly deprived of their usual energy source, glucose and O_2. They were exposed to a KHB solution from which glucose was deleted and which was saturated with a mixture of 95 percent N_2 and 5 percent CO_2. The signal increased from a steady value of 2.1 to 2.6 nA within 10 minutes and then slowly reached 6.0 nA after 90 minutes. When glucose and O_2 were reintroduced into the perfusing medium, the signal rapidly decreased and returned to the resting levels. A second exposure showed that this effect is quite reproducible. In the experiment illustrated in Fig. 3, another cell preparation was exposed several times to the calcium ionophore A23187. In a first 1.5-minute exposure to A23187 (1 μg/ml), the signal increased from 1.6 to 7.6 nA. Removal of the ionophore from the perfusion medium quickly reversed the effect. After a second, shorter exposure of 30 seconds with the same concentration of ionophore, the signal increased to 3.4 nA, and, after a final 10-minute exposure, it increased to approximating 7.1 nA. In all cases, the signal returned to baseline although the time necessary for this reversal was greater with longer exposures to the ionophore.

The method of Allen and Blinks for aequorin calibration was used to estimate the resting Ca_i^{2+} of cultured kidney cells incubated in KHB with 1.3 m*M* calcium (*7*). The observed signal was interpolated on a calibration curve relating the intensity of the signal to the concentration of free calcium. Since the intensity of the aequorin luminescence (*L*) depends on the total amount of aequorin present (L_{max}), the signal is normalized as a ratio L/L_{max}. For the cali-

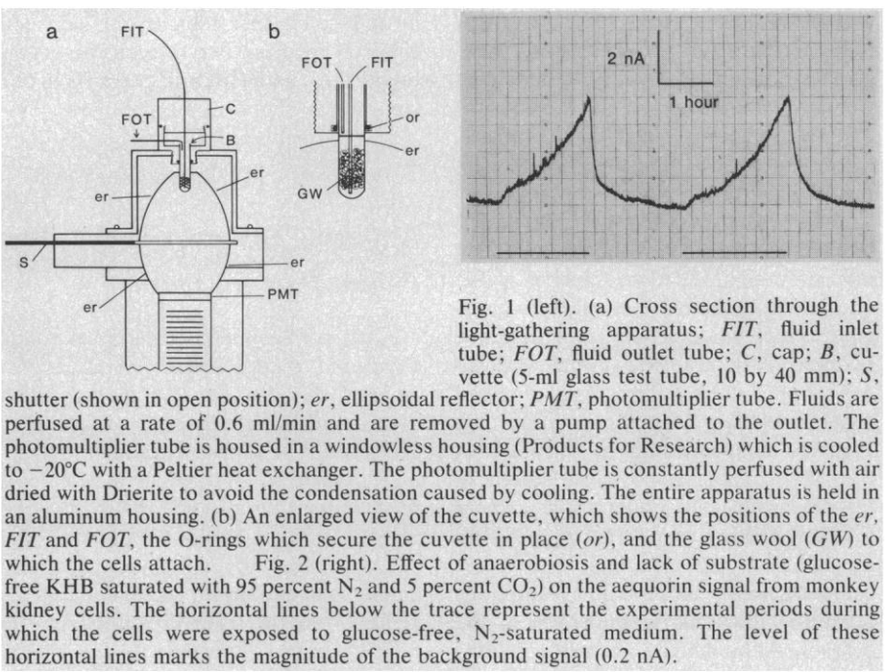

Fig. 1 (left). (a) Cross section through the light-gathering apparatus; *FIT*, fluid inlet tube; *FOT*, fluid outlet tube; *C*, cap; *B*, cuvette (5-ml glass test tube, 10 by 40 mm); *S*, shutter (shown in open position); *er*, ellipsoidal reflector; *PMT*, photomultiplier tube. Fluids are perfused at a rate of 0.6 ml/min and are removed by a pump attached to the outlet. The photomultiplier tube is housed in a windowless housing (Products for Research) which is cooled to −20°C with a Peltier heat exchanger. The photomultiplier tube is constantly perfused with air dried with Drierite to avoid the condensation caused by cooling. The entire apparatus is held in an aluminum housing. (b) An enlarged view of the cuvette, which shows the positions of the *er*, *FIT* and *FOT*, the O-rings which secure the cuvette in place (*or*), and the glass wool (*GW*) to which the cells attach. Fig. 2 (right). Effect of anaerobiosis and lack of substrate (glucose-free KHB saturated with 95 percent N_2 and 5 percent CO_2) on the aequorin signal from monkey kidney cells. The horizontal lines below the trace represent the experimental periods during which the cells were exposed to glucose-free, N_2-saturated medium. The level of these horizontal lines marks the magnitude of the background signal (0.2 nA).

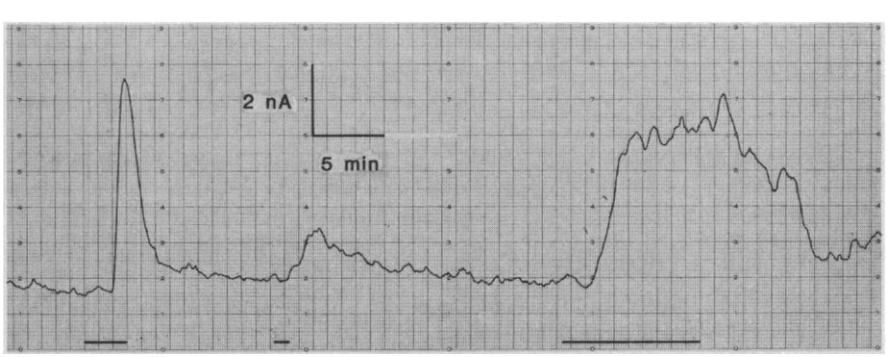

Fig. 3. Effect of the calcium ionophore A23187 (1 μg/ml) on the aequorin signal from monkey kidney cells. The ionophore was added to the perfusate during the time marked by the horizontal lines below the trace. The level of these horizontal lines marks the magnitude of the background signal (0.2 nA).

bration curve, the L observed at a known concentration of free calcium in a solution approximating the intracellular milieu (140 mM KCl, 2 mM Mg^{2+}, pH 7.4, 25°C) is divided by L_{max}, the maximum signal obtained when the same amount of aequorin is briskly injected into a solution containing a saturating concentration of calcium (10 mM). The ratio L/L_{max} is called the fractional luminescence. The titration curve is sigmoidal when drawn as a double logarithmic plot with pCa ($-\log Ca^{2+}$) as the abscissa and pF ($-\log L/L_{max}$) as the ordinate. The coefficient of variance is 1.6 percent. Similar aequorin luminescence titration curves have been published (*4*, *7*). In our experiments, L/L_{max} was determined from the observed signal minus background (L) divided by L_{max} (which is an estimate of the amount of aequorin incorporated in the cells) obtained when the cells were lysed at the end of the experiment with a solution containing 10 percent Triton-X, 10 mM $CaCl_2$, 140 mM KCl, 3 mM Hepes (pH 7.4), and 2 mM $MgCl_2$. The membrane detergent Triton-X does not interfere with the aequorin-calcium reaction, and, since the cell surface membrane is destroyed chemically, the optical geometry of the system is not disturbed. The luminescence evoked by Triton-X (L_{max}) was five to six orders of magnitude larger than the signal L, an indication that the consumption of aequorin during the experiments was negligible. An estimate of Ca_i^{2+} was obtained by interpolation of the experimental values on the calibration curve obtained with 2 mM Mg^{2+} in the solution, the assumed concentration of intracellular free Mg^{2+}. Table 1 shows the results of ten separate cell preparations where Ca_i^{2+} ranged between 16 and 90 nM with a mean of 57 nM. The validity of these estimates rests on several assumptions regarding the ionic composition of the cytosol. Although the activities of ions in the cytosol of monkey kidney cells are unknown, those chosen for the calibration curves (that is, 140 mM KCl, pH 7.4, 2 mM Mg^{2+}, 3 mM adenosine 5′-triphosphate) are close enough to provide an initial approximation for Ca_i^{2+}.

We investigated whether the HOST method altered the viability and the physiological properties of the cells. We found that HOST cells excluded the dye trypan blue as well as control cells. Their O_2 consumption was not stimulated by succinate (*8*). There was no difference between HOST cells and controls with respect to their total calcium or the size of calcium pools and fluxes measured by ^{45}Ca steady-state kinetics (*9*). The HOST cells could reestablish their normal ionic gradients for sodium and potassium even if loaded with 140 mM NaCl instead of KCl during the resealing after aequorin incorporation, and, if reinoculated into culture, HOST cells grew at a rate identical to that of control untreated cells.

We have developed a method by which a sufficient amount of aequorin can be incorporated into small mammalian cells to provide a measurable signal which reflects changes in Ca_i^{2+}. Its chief advantages are the following: (i) the method is relatively simple; (ii) the procedure is relatively inexpensive; (iii) with this method, a system is provided in which extracellular solutions may be perfused and changed without disturbing the cells; (iv) the viability and the physiological properties of the cells appear to be left unaltered; and (v) aequorin can be incorporated into large quantities of isolated cells, which produce an adequate signal and a good statistical representation of a cell population. The method can be applied to other cells: obelin has been similarly incorporated into pigeon erythrocytes (*10*) and in hybrid cells (*3*). In our laboratory, aequorin has been successfully incorporated into freshly isolated rat hepatocytes; their resting Ca_i^{2+} has been estimated to be 50 nM.

A. B. Borle
K. W. Snowdowne
Department of Physiology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261

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Antigenic Diversity in the Human Malaria Parasite *Plasmodium falciparum*

Abstract. *Monoclonal antibodies against blood forms of* Plasmodium falciparum *were used to demonstrate considerable antigenic diversity in this species. Different isolates were distinguished by their ability to react with certain antibodies, and most of the antibodies reacted specifically with merozoites, schizonts, or both. The distribution of different antigenic types appeared not to be related to geographic origin. Serological typing with monoclonal antibodies extends the range of methods for identification of different strains of this malaria parasite.*

Knowledge of antigenic diversity among populations of malaria parasites is important in research aimed at the development of a vaccine against malaria. Immunity to the blood forms of the parasite is, at least in part, directed against antigens of merozoites and schizonts (*1–4*), and since the immunity to *Plasmodium falciparum* may be strain-specific (*5–7*), these antigens seem likely to vary. The soluble S antigens present in the serum of patients infected with *P. falciparum* show extensive serological diversity (*8*), although the role of these antigens in protection is not clear and it is not known whether they are associated with different developmental stages of the parasite. The existence of stage-specific antigens associated with blood forms of *P. falciparum* has been shown by the use of monoclonal antibodies, some of which inhibit the growth of the parasite in culture (*3*). We have used monoclonal antibodies to investigate antigenic heterogeneity in *P. falciparum* isolated in different parts of the world, and here demonstrate that some antigens of merozoites and schizonts exhibit considerable diversity.

Monoclonal antibodies were prepared in mice against two *P. falciparum* isolates, K1 and PB1, from Thailand. The parasites to be used for immuniz tion were grown in vitro (*9*) and includ d all stages that occur in the blood, th t is, ring forms, trophozoites, schizonts, mer-ozoites, and gametocytes. Hybrid my-elomas were made by using a moc fication of the protocol of Perrin *et a* . (*3*) (see legend to Table 1). Those prod cing antibodies against parasites were i entified by indirect immunofluorescen e assay (IFA) (*10*) on acetone-fixed s ears of cultured blood forms. In seven f sion

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